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In vitro and in vivo evaluation of curcumin loaded lauroyl sulphated chitosan for enhancing oral bioavailability

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ABSTRACT

Curcumin has been demonstrated as a potent anticancer agent but its clinical application has been limited by its poor aqueous solubility and bioavailability. Here we describe encapsulation of curcumin in the lauroyl sulphated chitosan with a view to improve its bioavailability. In vitro antioxidant activity of extract of curcumin loaded matrix was investigated and exhibited dose dependent radical scavenging and reducing activity. Cytotoxicity studies carried out with curcumin loaded carrier on C6 cell line and were found to be toxic. Its in vitro effects on proliferation using the C6 cell lines also studied and observed antiproliferation of C6 cell line. Plasma concentration of curcumin–time profiles from pharmacokinetic studies in rats after oral administration showed a 11.5-fold increased pharmacological availability of curcumin with encapsulated curcumin compared with native curcumin. Overall we demonstrate that the curcumin loaded matrix has shown a superior pharmacological availability in vivo over curcumin.

1. Introduction

Recently rapid increase in the costs for health care has facilitated the importance of naturally occurring phytochemicals in plants for the prevention and treatment of human diseases, including cancer. Phytotherapy has been used for thousands of years all over the world and has been an important part of ancient culture in countries such as India and China. In recent decades the use of phytotherapeutics growing significantly among patients and physicians as is evident from an increased market for herbal medicines (Solecki & Shanidar, 1975). The global market for herbal medicines is expected to be $26 billion by 2012 (Saklani & Kutty, 2007). Most of the conventional chemotherapeutic agents used were designed to hit a single intracellular target. But the physiological and mechanistic deregulations responsible for cancer initiation and promotion caused by a hundreds of genes or signaling cascades so that it appears evident that multi-target drugs are requested to overcome complex human diseases such as cancer. Curcumin, an active chemical component issued from the plant Curcuma longa, exhibits a broad range of activities due to its ability to affect multiple intracellular targets and can be used as a multiple therapeutic agent.

Curcumin (CUR), polyphenolic compound, has emerged worldwide as a potent therapeutic substance for various human diseases including cancer and displays a wide range of pharmacological properties. In vitro and in vivo evaluation of curcumin indicated that it induces chemopreventive and chemotherapeutic effects on various cancer cell types and animal models (Huang et al., 1994; Nautiyal et al., 2011). Because of its low availability in the plasma, its therapeutic usefulness has been somewhat limited, leading researchers to investigate the benefits of complexing curcumin with other substances like natural polymers to increase systemic bioavailability. Even though the curcumin possess impressive array of medical benefits, the effectiveness of oral curcumin application has been limited by poor absorption into the bloodstream through the digestive tract.

But there is a medical need for improving the oral bioavailability of various poorly bioavailable curcumin because a major portion of a dose never reaches the plasma or exerts its pharmacologic effect. Hence to improve the oral bioavailability is therapeutically important because the extent of bioavailability directly influences plasma concentrations, as well as the therapeutic and toxic effects, resulting after oral drug administration. The most common approach for oral curcumin delivery is the encapsulation of drug using mucoadhesive polymers like chitosan by which the drugs is physically protected from enzymatic degradation. Moreover the controlled release formulations based on natural polymers like chitosan show numerous advantages, including protection of the drug over an extended period from degradation or elimination, biocompatibility, biodegradability, nontoxicity, etc. (Jayakumar, Menon, Manzoor, Nair, & Tamura, 2010). Drug molecules can be incorporated within

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the polymer after functionalization and structural manipulation of polymer material and the advantages of entrapping curcumin within a polymer allows for greater control of the pharmacokinetic behavior of the active drug molecule.

A number of amphiphilic systems have been reported for the effective curcumin delivery. Dextran sulphate–chitosan nanoparticles was found to be a good matrix for the curcumin delivery to the tumor cell line and curcumin loaded these matrix showed preferential killing of cancer cells compared to normal cells, indicating potential in targeting (Anitha, Deepagan, et al., 2011). Anitha, Maya, et al. (2011) synthesized curcumin-loaded O-carboxymethylchitosan nanoparticles as a novel carrier in cancer drug delivery applications and these drug loaded particles was found to be toxic to the cancer cell lines like MCF-7, PC-3, etc. Cellular internalization of particles was also observed after the incubation of cells with these particles.

We have reported the development, characterizations and some in vitro evaluation of lauroyl sulphated chitosan (LSCS) and its in vivo evaluations of insulin encapsulated LSCS submicroparticles (Shelma & Sharma, 2011a,b, 2013).

Here we report the in vivo efficacy of the orally delivered curcumin loaded lauroyl sulphated chitosan (LSCS-CUR) submicroparticles of size 629.5 ± 20.5 nm and zeta potential of -6.06 ± 1.22 for improving its bioavailability.

2. Materials and methods

Chitosan was purchased from Indian Sea Food (Cochin, India) and used after purification (purified CS has molecular weight of 270 kDa). Curcumin, sodium nitroprusside, potassium ferricyanide, ferric chloride, ascorbic acid, dimethyl thiazolyl diphenyl tetrazolium salt (MTT), and agar were purchased from Sigma–Aldrich chemicals. C6 cells were purchased from National Center for Cell sciences Pune, India. All other chemicals were of analytical grade from Merck and used as received.

2.1. Preparation of LSCS-CUR

We have developed the matrix LSCS by the reaction between chitosan and sulphobenzoic acid anhydride followed by the treatment with lauroyl chloride through acid chloride route. Its physicochemical properties and some biological properties were reported (Shelma & Sharma, 2011a,b). LSCS product is then dissolved in acetic acid solution (1%) to get 0.25% solution and the CUR in acetone was added after ultrasonication. Stirred magnetically and then added tripolyphosphate (0.25%) for the preparation of LSCS-CUR submicroparticles of size 629.5 ± 20.5 nm through ionic gelation method.

2.2. FTIR

Surface characterizations of CUR and LSCS-CUR were performed using spectroscopic technique Fourier transform infrared spectroscopy with attenuated total reflectance (ATR–FT-IR).

2.3. Differential scanning calorimetry (DSC)

The thermal properties of CUR and LSCS-CUR were studied by DSC where thermograms were performed on a Q 20 DSC thermal analyzer (TA Instrument, USA) with an auto cool accessory. Each sample weighed approximately 5 mg encapsulated in an aluminum pan and was heated from 25 °C to 400 °C at 10 °C/min under a nitrogen atmosphere. An empty loosely covered aluminum pan was used as the reference.

2.4. AFM

Surface morphology of the CUR, LSCS and LSCS-CUR was studied using AFM (JEOL JSPM-5200).

2.5. Water solubility study

To compare the solubility of CUR before and following the encapsulation process, solubility of CUR in water was determined. CUR (10 mg) and LSCS-CUR particles which contain an equivalent amount of CUR (10 mg) were added to 1 ml of deionized water. Mixtures were vortexes for 5 min; sonicated in water for 1 min, and centrifuged at 10,000 rpm for 5 min. Supernatants were mixed with ethyl acetate (1:1, v/v) to CUR extract. Finally, CUR concentration was determined spectrophotometrically at 420 nm.

2.6. In vitro antioxidant activity

2.6.1. Assay of NO radical scavenging activity

This assay was performed according to the method described elsewhere (Sreejayan & Roa, 1997). Sodium nitroprusside in aqueous solution at physiological pH produce nitric oxide which interacts with oxygen to produce nitrite ions, which was measured by Griess reagent. The reaction mixture containing 1 ml of 10 mM sodium nitroprusside in phosphate buffered saline and 1 ml of CUR extract in methanol at different concentrations (5–40 μM) were incubated at 37 °C for 150 min. About 1 ml aliquot of the incubated sample was mixed with 1 ml of Griess reagent and the absorbance of the chromophore formed was measured at 546 nm. Inhibition of the nitric oxide generated was measured by comparing the absorbance values of control that is the sodium nitroprusside without the samples.

The nitric oxide radicals scavenging activity was calculated according to the following formula:

$$\%\,\text{Inhibition} = \left(\frac{(A_0 - A) \times 100}{A_0}\right)$$

where A_0 was the absorbance of the control that is without CUR extract and A was the absorbance in the presence of the extract.

2.6.2. Reducing power by $FeCl_3$

The reducing power of CUR was determined according to the method previously described (Oyaizu, 1986). Different concentrations of CUR extract in methanol (10–100 μM) was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50 °C for 30 min. 2.5 ml of 10% trichloroacetic acid was added to the incubated mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml distilled water and 0.5 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm.

2.7. Cytotoxicity studies on C6 cell lines

Cytotoxicity of LSCS and LSCS-CUR were carried out on C6 cell line (fibroblast glioma cell line derived from rat tissue) by Dimethyl thiazolyl diphenyl tetrazolium salt (MTT) assay. C6 cells were cultured in DMEM/Ham's F12:MEM (1:1) medium supplemented with 10% FBS for C6 cells at 37 °C using a humid 5% CO_2 incubator. Particles (LSCS and LSCS-CUR) were added to the wells containing C6 cells in duplicate and incubated for 24 h. After the incubation period, the medium containing vesicles were removed and 200 μl of MTT (0.5 mg/ml) was added, incubated for another 3 h at 37 °C. The formazan crystals formed were dissolved in 250 μl DMSO and read the absorbance at 570 nm. The cells treated with medium alone were used as negative control and cells treated with 1%

Triton-X-100 were used as the positive control. Experiment was carried out four times.

2.8. Live and dead assay

C6 cells were seeded into 4 well plates and allowed to adhere with 5% CO_2 at 37 °C. The cells were incubated with the CUR (20 μM) and LSCS-CUR (20 μM) for 3 h, 24 h and 48 h and the cells without particles was the control. The live and dead assay was performed using the Live and Dead kit protocol. The cells were washed with phosphate buffered saline three times. Then freshly prepared 200 μl solution of calcein AM/EthD-1 reagent in phosphate buffered saline added to the cells, which were further incubated for 30 min at 37 °C. The treated cells were then visualized using fluorescence microscope (Leica DM IRB, Germany).

2.9. Soft agar colony assay

To know how the curcumin affects the tumorigenicity of C6 cells in vitro, soft agar colony formation was performed. Each 75 mm autoclaved culture dish contained a base layer consisting of 1% agarose (final concentration of 0.5%) in media and allowed to solidify. Suspension of C6 cells, which harvested from monolayer culture and molten 0.7% agar (to a final concentration of 0.35%) with void polymer, free curcumin or LSCS-CUR poured to the base layer and allowed to set. Medium was replaced 2 days intervals and cultures were placed in cell culture incubators at 37 °C, 5% CO_2 for 6 days. The colony assay was terminated and stained with 0.005% crystal violet. The picture of colonies observed in the soft agar culture with control, native curcumin and LSCS-CUR were captured.

2.10. Animal studies

The importance of in vivo models is the integration of the dynamic components of the blood circulation, the mucous layer and all other factors that can influence drug dissolution. Mainly used animal model is rat, since it better reflects the human situation with respect to metabolism and paracellular space (Kararli, 1995). We have used male Sprague–Dawley (SD) rats for the study.

2.10.1. Bioadhesion experiment using texture analyzer

Bioadhesion measurement is based on the principle of recording force required to break the adhesive bond between a model membrane and the test formulation. Bioadhesive strength of the gel was measured using a calibrated texture analyzer (TA.XT plus texture analyzer, Stable Micro Systems, UK) equipped with a 50 kg load cell with mucoadhesive holder. CS, LSCS and LSCS-CUR submicroparticles were attached to the cylindrical probe (10 mm in diameter) with the help of double-sided adhesive tape. The mucosal tissues of SD rat were cut in small piece (2 cm × 2 cm) and cleaned using chilled phosphate buffer saline. The rat intestinal tissue placed tightly onto the mucoadhesive holder using clips and the particles attached the probe were then moved downward to contact with the rat tissue at a specified force and maintained until specified time in order to establish a proper contact between the membrane and sample, and to allow the formation of an adhesive bond. The probe was subsequently withdrawn at a specified test speed. Force required to detach the two membranes was measured directly using Texture Exponent 32 software analyzer. Maximum force (i.e. maximum detachment force; F_{max}) required to break the bond was measured as bioadhesive strength and area under curve was measured as work of adhesion. For all the formulations, mean bioadhesive strength and work of adhesion (W_{ad}) were determined in replicates ($n = 5$) and the mean values determined.

2.10.2. Biodistribution of curcumin

SD rats were fastened for 16 h prior to the experiment. To determine the biodistribution of curcumin, LSCS-CUR particles in saline were orally administered to SD rats by oral gavage. Rats were euthanized after 2 h, 1 day and 2 day and tissue samples like duodenum, jejunum, ileum, liver, kidney, and spleen portions were cut longitudinally and frozen suddenly at −70 °C. The samples were then cut into 6 μm sections by a microtome (Crytome E; ShanDon, England) at −20 °C. After being mounted on a glass slide, the sections were subjected to dehydration in the sections by dipping for 2 min each at formalin solution, ethanol solutions subsequently (70%, 80%, 90% and 100%) and finally in xylin. Thereafter, the sections were observed using a fluorescence microscope (Leica DMIRB, USA).

2.10.3. Extraction of curcumin from tissue samples

To extract curcumin from the LSCS-CUR that had attached to the stomach tissues, liver, spleen and kidney, the freeze-dried tissue samples were accurately weighed and then soaked in 1.0 ml of ethyl acetate (overnight). The suspension was then vortexes and centrifuged at 8000 rpm for 10 min to pellet the solid. The supernatant liquid was collected, and dried. Dried samples then solvated in 500 μl of methanol and subjected to quantification of the curcumin content by HPLC.

2.10.4. Pharmacodynamic study

The different pharmacokinetic parameters of free curcumin and LSCS-CUR were determined in SD rats weighing between 220 and 250 g and animals were maintained in an environmentally controlled room (23 ± 3 °C, 12 h dark–light cycle) with free access to food and water before experiments. They were fasted overnight before the administration of curcumin and LSCS-CUR. Animal experiments were approved by the Animal Ethics Committee at Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, India. The rats for the experiment are divided into five groups and each group contains six rats. The first group was control and 1 ml normal saline was administered orally by using oral gavages. In the same way freeze dried LSCS-CUR particles (equivalent to doses of 10 mg curcumin/kg weight of rat and 50 mg/kg) were administered orally to the groups 2 and 3 after dispersed in 1 ml of normal saline. Fourth group has received the curcumin intravenously equivalent to 10 mg/kg.

The relative oral bioavailability of LSCS-CUR formulations was determined by oral administration of the free curcumin to the rats of fifth group. After administration, blood samples were collected (500 μl) from the retro-orbital plexus at 0.5, 2, 4, 6, 24, 48, 72, 96, 120, 144, and 164 h in the heparinized micro centrifuge tubes (100 IU heparin/ml of blood). The plasma was separated by centrifuging the blood at 4000 rpm for 5 min. The curcumin concentration in the plasma samples was then subjected to curcumin extraction and quantification by HPLC method (Shimadzu LC-2010 A HT). Separation was achieved on a C-18 column using HPLC grade methanol as the mobile phase with a flow rate of 20 μl/min.

The standard curve for curcumin was plot using HPLC and it was linear with a correlation coefficient of $R^2 = 0.99$.

3. Results and discussions

3.1. FTIR

The interaction between curcumin and LSCS is confirmed with FTIR (Supplementary Fig. 1). Supplementary Fig. 1 shows the FTIR spectra of curcumin, LSCS-CUR. The most prominent peaks for curcumin are as follows (cm^{-1}): 3504, 1626, 1600, 1560, 1508, 1458, 1427, 1314, 1273, 1231, 1204, 1181, 1151, 1114, 1025, 977, 961, 885, 855, 807, 713, and 668 cm^{-1}. Generally the stretching frequencies correspond to hydroxyl group (O—H) lies in the band

range of 3200–3600 cm^{-1}. The band for carbonyl group (C=O) peaks appeared at the band range of 1620–1650 cm^{-1} and of alkanes (C—H) is shown at 1350–1512 cm^{-1}. Curcumin showed a characteristic intense peak of hydroxyl group at 3504 cm^{-1}. The band at around 1025 cm^{-1} characterized the vibration of C—O stretching band for curcumin. The wave number for curcumin was noticed at 1508 cm^{-1} which correspond to the C—H stretching.

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LSCS-CUR shows the FTIR peaks at 3246, 2916, 2848, 1698, 1628, 1535, 1464, 1429, 1377, 1248, 1153, 1069, 1027, and 894 cm^{-1}. In LSCS-CUR, a broad peak for hydroxyl group appears at 3240 cm^{-1}, which shows that some interaction such as the intermolecular hydrogen bonding between curcumin and LSCS has occurred at hydroxyl (—OH) group. This interaction caused to change the crystalline structure to amorphous form. Same way the stretching band corresponds to C—H shifted to a higher wave number of 1600 cm^{-1} indicated the participation of C—H group in the strengthening the complexation. This type of interaction may cause to change curcumin crystalline structure to amorphous form. FTIR spectroscopy thus elucidates the interaction between curcumin and LSCS.

3.2. DSC

DSC thermograms of curcumin and LSCS-CUR are illustrated in Supplementary Fig. 2. DSC curve of pure curcumin showed a sharp endothermic peak at 177 °C, corresponding to the melting point of curcumin. In the case of LSCS-CUR the DSC curves showed broad endotherms with peak temperature slightly shifted to lower temperature than pure curcumin. These findings may be due to the formation of some interaction between curcumin and LSCS matrix. This characterization also confirms the hydrophobic–hydrophobic interaction.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.02.029.

3.3. AFM

Surface morphology of particles was observed with AFM and is shown in Fig. 1. AFM images show that the average size of the particles is around 500–600 nm with spherical morphology. From the figure it is also clear that the prepared NPs have a smooth surface with spherical morphology.

3.4. Water solubility study

To verify the solubility of curcumin before and after encapsulation procedures, we dissolved curcumin and LSCS-CUR in aqueous solution and the dissolved concentrations were determined spectrophotometrically. Enhanced solubility of curcumin is shown in Supplementary Fig. 3. From the figure we can see the macroscopic flakes of curcumin which is poorly soluble in aqueous medium. In contrast LSCS-CUR solution provided a clear, well dispersed formulation with curcumin natural color. The pure curcumin is insoluble in water because of its hydrophobicity that caused the powder to float on the surface of water. The solubility of curcumin in water was 4.375 μg/ml, where as that of LSCS-CUR was 56.6639 μg/ml, which represented a 13-fold, increase. The significant increase in its solubility was observed from the LSCS-CUR. We can suggest that the LSCS might form a soluble complex with the curcumin. The main reason for the low oral bioavailability of curcumin is its insolubility in aqueous solutions and many of the researchers may be interested in how to improve solubility of curcumin. We can say that the increase of curcumin dissolution from these LSCS matrix may due to several factors such as an excellent wettability, that observed from the solid dispersion since it rapidly left the surface of the solid

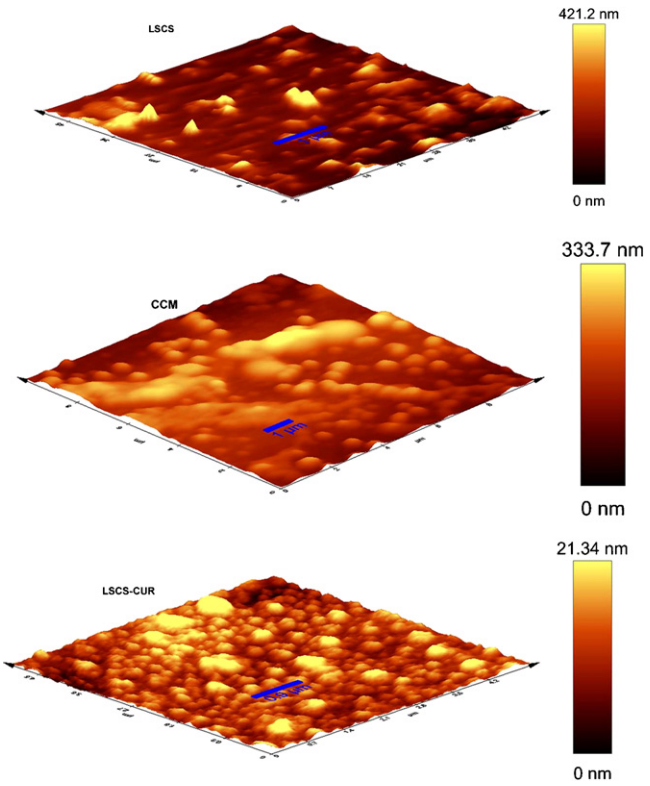

Fig. 1. AFM images of LSCS particles, CUR, and LSCS-CUR.

LSCS matrix and was dispersed in the bulk of the water, a markedly increase in curcumin solubility, the solubilizing effect of the carrier and the conversion of crystallite to amorphous as confirmed by this study.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.02.029.

3.5. In vitro antioxidant activity

The reducing power and free radical scavenging activity of curcumin is well known. Thus, it was of interest to see if the encapsulated curcumin in LSCS matrix preparations still possesses such activity. To this end, here, the scavenging of nitrite radicals and reducing of iron also were monitored, and the LSCS-CUR particles showed obvious reducing and radical scavenging activity.

3.5.1. Assay of NO radical scavenging activity

In vitro inhibition of nitric oxide radical, a free radical which plays an important role in the pathogenesis of pain, inflammation, etc., is a measure of antioxidant activity of the material. The results of the free radical scavenging potentials of the extracts of curcumin from LSCS-CUR tested by nitric oxide scavenging method are depicted in Fig. 2A. With the increase of curcumin concentrations from LSCS-CUR effectively reduced the generation of nitric oxide from sodium nitroprusside that means a strong nitric oxide scavenging activity. The current study indicates that curcumin extract effectively scavenged nitric oxide radicals in vitro (Fig. 2A). This radical are generated inside the body during the normal metabolism or in presence of xenobiotics. With the increase of concentrations of curcumin extract from LSCS-CUR decreased the amount of nitrite generated from the decomposition of sodium nitroprusside in vitro. This observation may be due to the antioxidant component in the extracts which compete with oxygen to react with NO free radical and thereby inhibiting the generation of nitrite.

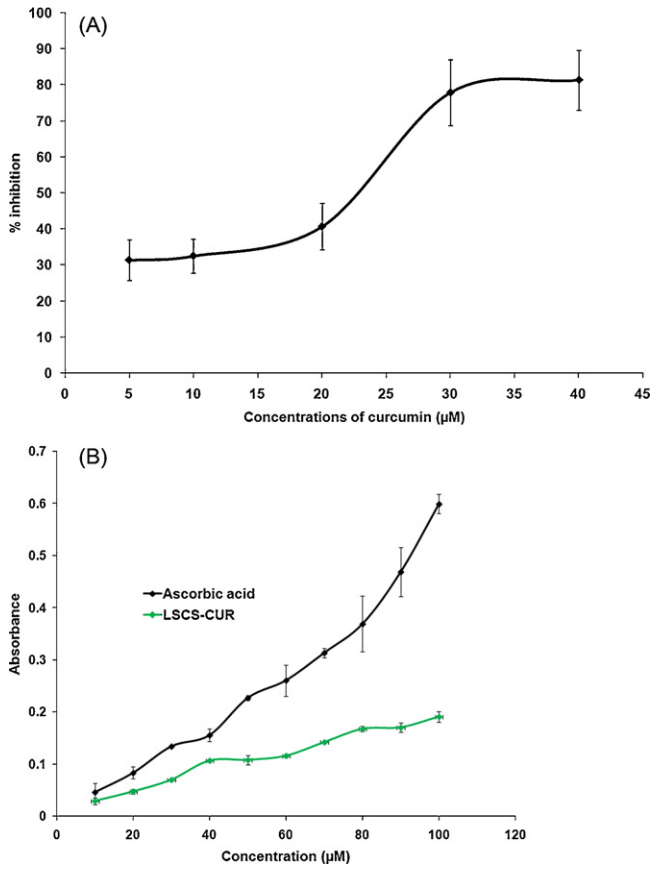

Fig. 2. (A) Nitric oxide scavenging potential of methanol extract of curcumin from LSCS-CUR at different concentrations (μg/ml). Values are the average of triplicate ± standard deviation; (B) Reducing power of the curcumin extract from LSCS-CUR compared to ascorbic acid. Values are the average of triplicate ± standard deviation.

3.5.2. Reducing power by $FeCl_3$

Reducing power assessment is based on the ability of antioxidants to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}). The ability of a compound to carry out the reduction of iron is a significant indicator of its antioxidant activity (Barzegar et al., 2011). The antioxidant activity of the methanol extracts of the curcumin was investigated by using reducing power of the extract and by determining antioxidant capacity of the extract. This experiment has proven the effectiveness of the methanol extract compared to the reference standard antioxidant ascorbic acid.

Reducing power of the curcumin extract exhibited concentration dependent increase in absorbance (Fig. 2B). Increased absorbance of the reaction mixture indicated increased reducing power due to formation of reduced intermediate. The absorbance is increased with increase of concentration that means an existence of a good linear relationship between curcumin concentrations and reduced amounts of Fe^{3+} ions. This method applied to determine and analyze the electron donating potency of curcumin. Ascorbic acid was used as a reference standard of the same concentrations. Phosphate buffer (pH 6.6) was used as blank solution. The absorbance of the final reaction mixture of two parallel experiments were taken was expressed as mean ± standard deviation. The reducing ability of a material generally depends on the presence of reductants (Duh, Tu, & Yen, 1999). The reductants exhibited antioxidative potential by breaking the free radical chain, donating a hydrogen atom. The presence of reductants in the CUR extract causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form and the ferrous could be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Fig. 2B shows the reductive capabilities of the curcumin extract compared to ascorbic acid. The reducing power of curcumin extract was very potent and the power of the extract was increased with quantity of sample. The sample extract could reduce the most Fe^{3+} ions, which had a lesser reductive activity than the reference standard of ascorbic acid.

We can explain the reason of higher reducing potency of curcumin based on its unique structure. Curcumin has a conjugated structure including two methoxy phenols and a diketone. Methoxy phenolic group present in the curcumin act primarily as hydrogen atom donators while diketone act as electron donors, leading to chain-breaking reactions of radicals. Hence curcumin shows a higher reducing capability because of its unique structure and functional groups, including a diketo group and phenyl rings that contain more p-electrons which can fully conjugate between benzene units result a high reducing capability.

Table 1
Percentage viability of C6 cells after 24 h incubation with the particles LSCS and LSCS-CUR ($n = 4$).

Sample	% viability
LSCS	66.93 ± 0.02
LSCS-CUR	30.47 ± 3.4

3.6. Cytotoxicity

Percentage of viability after the incubation of the particles with C6 is demonstrated in Table 1. The results indicate cytotoxic effects of LSCS-CUR toward C6 cell line while unloaded particles were non-toxic to the C6 cell lines. The cytotoxicity studies on C6 cell lines support the previously reported observation that curcumin shows toxicity to tumor cells while the bare particles is found to be non-toxic. This shows the significance of loaded curcumin in the LSCS matrix toward the anticancer therapy (Table 1).

3.7. Live and dead assay

This is a two color fluorescence assay which used to measure cell viability. The live and dead assay evaluates the cell membrane permeability due to cell death. Live cells have intracellular esterase which convert the nonfluorescent, cell-permeable calcein AM to the intensely fluorescent calcein and are retained within cells. In the case of dead cells which have damaged membranes, the EthD-1enters damaged cells and produces a bright red fluorescence when bound to nucleic acids. Live and dead assay of C6 cells after incubation of 3 h, 24 h and 48 h with bare LSCS, curcumin (20 μM), LSCS-CUR (20 μM) is picturized in Fig. 3. From Fig. 3, a significant cell death was observed for curcumin and LSCS-CUR samples after 24 h and 48 h of incubation time of C6 cells with the samples. Cell death may be due to the effect of curcumin that rupture the cell plasma membrane at the end of incubation time. At the same time the control cells which was treated the same way was live after the incubation. From the study we could understand that these LSCS-CUR particles due to the incorporation of curcumin could produce the toxic effect of the matrix to the cancer glioma cell lines while untreated C6 cells were found to be active that means the absence of any toxicity.

3.8. Soft colony assay

Inhibiting the formation and proliferation of cancerous cells is important in cancer treatment. Colony soft agar assay is an anchorage independent growth assay in soft agar and it is used to determine the antiproliferative efficacy of curcumin that entrapped in the C6 cells. Colony assay in soft agar were performed by comparing the effect of free and encapsulated curcumin in inhibiting the clonogenicity of the C6 cell line. Antiproliferative effect of the curcumin loaded particles was demonstrated in Fig. 4

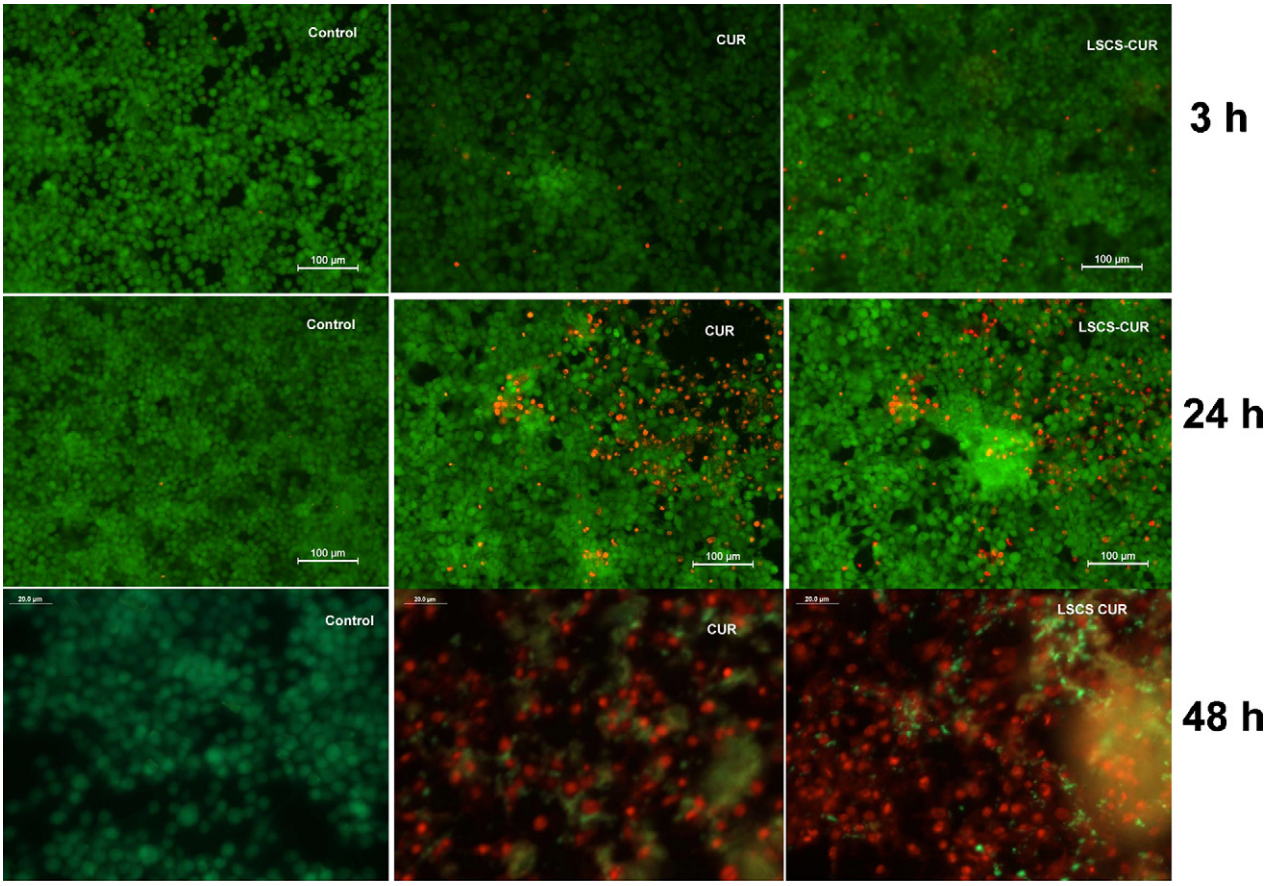

Fig. 3. Live and dead assay of control, CUR and LSCS-CUR using C6 cell lines.

compared to the control and bare curcumin. It was found that curcumin and LSCS-CUR inhibited colony formation and reduced the size of colonies but there was proliferation of C6 cells in the control petridish. The figure implies that LSCS-CUR could profoundly inhibit the proliferation of C6 cells at a dose of 20 μM in soft agar compared to the colony observed from curcumin treated cell and also explains its clinical importance for cancer treatment. This suggests that curcumin entrapped in LSCS matrix has a comparatively better antiproliferative ability as it was effectively blocked the clonogenicity of C6 cell compared to curcumin treated cell. This type of antiproliferative effect is mediated partly through the suppression of growth of this type of tumor cell line.

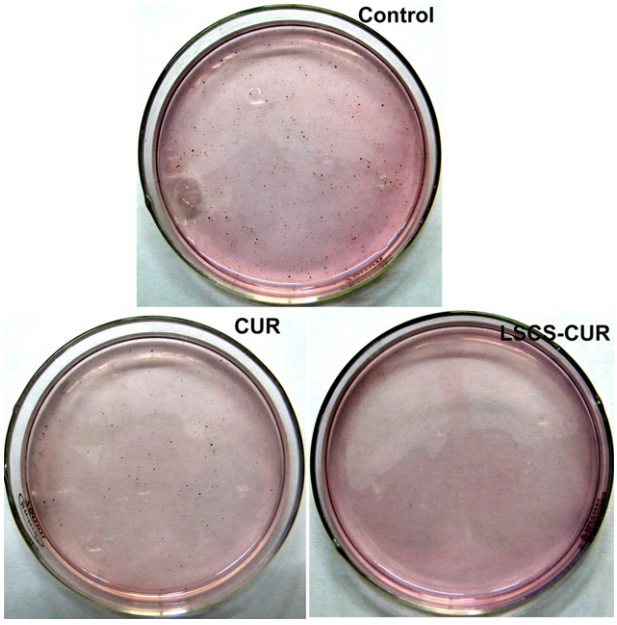

Fig. 4. Colony assays in soft agar of control, native CUR and LSCS-CUR.

3.9. Bioadhesion

In this study, the mucoadhesive effect of CS, LSCS and LSCS-CUR evaluated using the texture analyzer. Texture analyzer is a useful technique and has been extensively used as a means for mechanical characterization of pharmaceutical mucoadhesive dosage forms. Detachment force studies have been used as a direct measure of mucoadhesivity of a material (Pritchard et al., 1996). This parameter gives an idea about the force required to separate the surface of a mucoadhesive material from that of the mucoadhesive substrate that means the mucoadhesive strength of the material. This type of strength is considered to be dependent on the formation of hydrogen bonds between the functional groups of the bioadhesive particles (here OH groups in CS, hydrophobic moieties in LSCS and LSCS-CUR in addition to the OH groups) and the mucus membrane. In other words, the interaction between the mucosal surfaces and bioadhesive polymers occurs as a result of physical entanglement and secondary bonding, mainly hydrogen bonding, Van der Waals attractions and hydrophobic–hydrophobic interaction. CS shows a bioadhesive nature as we expected (Table 2). This detachment force increases for LSCS and LSCS-CUR compared to CS and the order of increase was CS < LSCS < LSCS-CUR. The enhanced mucoadhesive strength may be due to increase in more available adhesive sites of LSCS and LSCS-CUR.

Table 2
Detachment force and work of adhesion of the native and modified particles ($n = 5$).

Sample	F_{max}	W_{ad}
CS	0.5382 ± 0.009	0.3074 ± 0.007
LSCS	0.6628 ± 0.009	0.3785 ± 0.002
LSCS-CUR	0.9932 ± 0.039	0.5671 ± 0.009

3.10. HPLC quantification of curcumin

Curcumin present in the tissue samples and plasma samples was analyzed quantitatively by HPLC method using methanol as the mobile phase at a flow rate of 1 ml min^{-1}. The eluent was monitored with a UV/VIS detector at 420 nm. The average amount of curcumin in each sample was determined from replicate sample injections by integration of the peak area with the aid of a calibration curve, constructed from a series of curcumin standards in methanol.

3.11. Biodistribution

To investigate the biodistribution of LSCS-CUR in vivo, we administered LSCS-CUR particle to normal SD rats at a curcumin dose of 10 mg/kg. Biodistribution of the orally delivered LSCS-CUR in intestinal portions, liver, kidney and spleen after 2 h, 1 day and 2 days of administration were depicted in Fig. 5.

After 2 h of oral administration of LSCS-CUR to rats, curcumin concentration was found to be in normal tissues like duodenum, jejunum, ileum, liver, spleen, and kidney. In the case of last three tissues the order of curcumin is in decreasing. This is because liver is the main organ that uptake the curcumin than other organs. The intestine part showed a higher amount of curcumin for the first hours after the administration, which suggested an increase of intestinal bioadhesion. As time over, the curcumin concentration in the duodenum portion is decreasing and completely eliminated after 2 days. But in the jejunum portions we observed the presence of curcumin even after 2 days. The concentration of curcumin in the ileum is increasing after each day. This may enhance the bioavailability of curcumin.

3.12. Pharmacodynamic study

To characterize LSCS-CUR and its ability to play a role as a potential chemotherapeutic agent for cancer, we decided to give LSCS-CUR in rat and evaluate its in vivo stability. The in vivo studies give a clear idea about the ability of LSCS-CUR to attach to the mucosal layer of the GI tract and their ability to deliver curcumin into the blood circulation. For comparative studies, we included curcumin also in the in vivo experiments, a compound with known pharmacokinetic properties. The in vivo pharmacokinetics study shows that the SD rats administered with free curcumin showed a

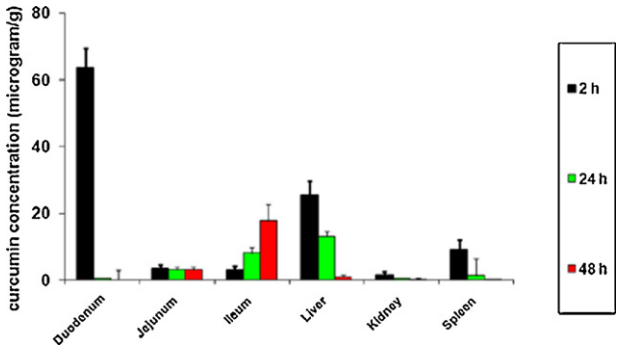

Fig. 5. Curcumin biodistribution in organs at different times (2 h, 1 day and 2 day) after oral administration of LSCS-CUR.

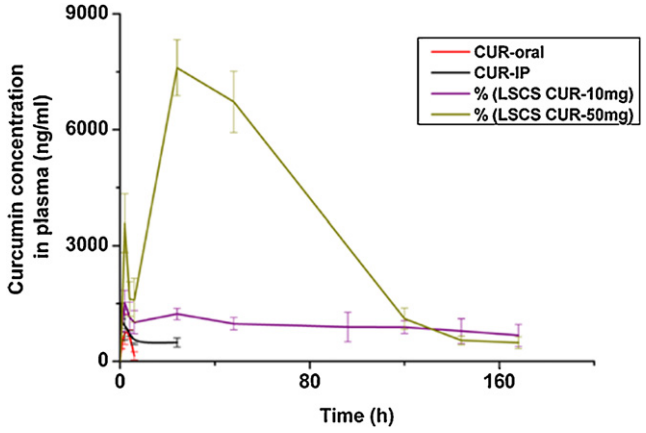

Fig. 6. Curcumin plasmatic profile after oral administration of native CUR (10 mg/kg), LSCS-CUR (10 mg/kg and 50 mg/kg) and intraperitoneal administration of native CUR (10 mg/kg).

significantly lower amount of curcumin in their blood than those administered with LSCS-CUR (Fig. 6). Concentrations of curcumin present in the plasma as a function of time following administration in rat are depicted in figure. Serum concentration of curcumin obtained from both the doses of LSCS matrix was higher than pure curcumin and the complex maintained effective concentration of curcumin for a longer period of time that is 7 days in rat serum but only 6 h for oral curcumin. In order to determine the oral pharmacological availability of curcumin, pharmacokinetics of native curcumin in rats has been conducted via intraperitoneal route.

Good contact between the particles and the intestinal surface would be an important factor and the texture analyzer studies showed excellent adhesion of the particles and tissue. It has been known that CS is a mucoadhesive polymer and we also found that LSCS is also a better mucoadhesive than chitosan and LSCS-CUR is more mucoadhesive than LSCS. The long stay of the LSCS-CUR particles on the stomach surface enabled the protected curcumin molecules in the particles to be continuously diffused from the particles directly into the tissue, and so a high curcumin concentration was observed in the blood for a long time.

The hydrophilic nature of their outer surface should make the particles dispersible in the mucous gel layer. Upon contact with the mucosa, hydrogen bonding between the particles and the mucin protein is very likely to occur and this interaction prolongs the time the curcumin loaded particles stay in the intestinal tract and facilitate the contact between curcumin loaded particles and the intestinal tissue. Upon contact with intestinal surface, direct diffusion of curcumin from the LSCS-CUR particles into the intestinal epithelium can occur and this matrix which are stuck at the intestinal surface act as reservoirs of curcumin. This matrix protects the curcumin from the degradation of curcumin and hence an increased bioavailability could be observed.

The hydrophobic curcumin molecules will quickly aggregated under the aqueous environment and quickly removed down the GIT very quickly. It has been already reported that curcumin possesses a rapid metabolism (Wahlstrom & Blennow, 1978) and fast elimination (Maiti, Mukherjee, Gantait, Saha, & Mukherjee, 2007; Sharma et al., 2001). Therefore, the prolonged sustainability of curcumin observed here implied a continuous supply of the curcumin into the circulation. curcumin concentration in the blood plasma of rat stays for 7 days in the case of both the doses of LSCS-CUR. But in the case of orally delivered bare curcumin shows the presence of curcumin blood only for 6 h and that of intraperitoneal administration of 24 h.

Bioavailability of curcumin could then be estimated by integrating amount of the curcumin present in the blood after administration. The pharmacological availability of curcumin in

blood was 0.2346 and 11.4475 for free curcumin and LSCS-CUR, respectively, compared with intraperitoneal administration. From these results we can say that about 48.79 times increased the pharmacological availability compared to the unencapsulated curcumin. Therefore, it could be concluded that the existence of curcumin in the blood was significantly longer for the matrix compared with free curcumin.

4. Conclusion

LSCS-CUR is subjected to many biological experiments and the results are summarized here. The antiproliferative effect of curcumin on C6 cell line enhances by curcumin loaded particles. LSCS-CUR was found to be toxic toward C6 cell line. LSCS-CUR bears a potent antioxidant activity as their constituents scavenge free radicals and have reducing activities. LSCS submicroparticles are promising candidates that will enable efficient in vivo delivery of oral curcumin for cancer treatment. When orally administered to rats, curcumin was observed in tissues confirming the well attachment of LSCS-CUR particles to the gastrointestinal mucosa. A single dose of LSCS-CUR particles sustained curcumin levels in the blood for 7 days and the pharmacological availability of curcumin is 11.5. LSCS-CUR circumvents the problem of low oral bioavailability of curcumin by protecting the drug from enzymatic degradation. The experiments in the current study provide a biologic rationale for treatment of patients suffering from carcinoma with this nontoxic phytochemical encapsulated LSCS for oral delivery.

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